



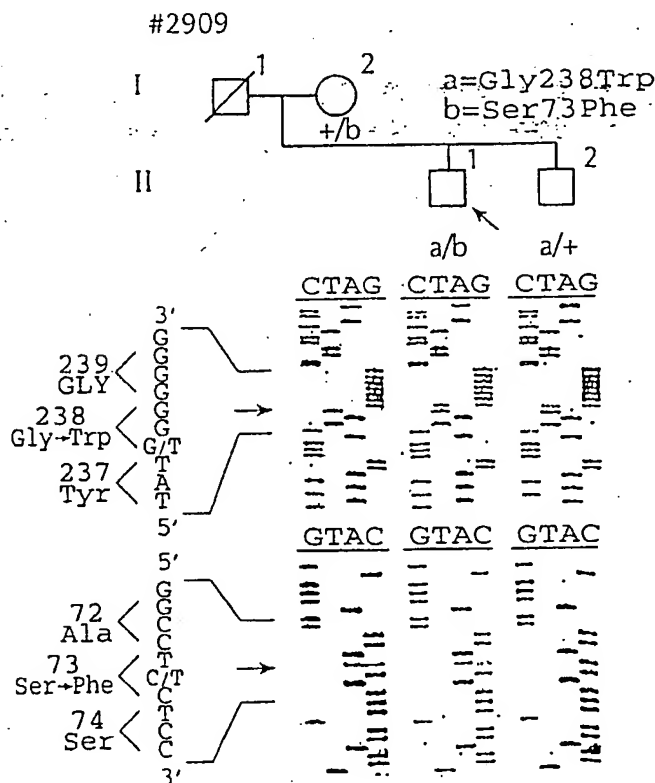
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(54) Title: MUTATIONS IN NUCLEIC ACID MOLECULES ENCODING 11-CIS RETINOL DEHYDROGENASE, THE MUTATED PROTEINS, AND USES THEREOF

(57) Abstract

The invention relates to mutations in the gene encoding 11-cis retinal dehydrogenase. The mutations in the gene and in the resulting encoded protein are correlated to ocular disorders, such as fundus albipunctatus.



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**MUTATIONS IN NUCLEIC
ACID MOLECULES ENCODING
11-CIS RETINOL DEHYDROGENASE,
THE MUTATED PROTEINS, AND USES THEREOF**

5

FIELD OF THE INVENTION

This invention relates to mutations in nucleic acid molecules encoding the protein 11-cis retinol dehydrogenase, or "RDH5," and the resulting mutated protein. These mutations are implicated in ocular disorders, such as fundus albipunctatus. The diagnostic and therapeutic ramifications of these mutations are also discussed and are features of the invention.

10

BACKGROUND AND PRIOR ART

Retinoids (vitamin A-derivatives) have important physiological functions in a variety of biological processes. During embryonic growth and development, as well as during growth and differentiation of adult organisms, retinoids act as hormones and participate in the regulation of gene expression in a number of cell types. See Lied et al. Trends Genet., 17:427-433 (1992). It is believed that these effects are mediated through two classes of nuclear ligand-controlled transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), Benbrook et al., Nature, 333:669-672 (1988); Brand et al., Nature, 332:850-853 (1988); Giguere et al., Nature, 330:624-629 (1987); Mangelsdorf et al., Nature, 345:224-229 (1990); Mangelsdorf, et al. Genes Dev. 6:329-344 (1992); Petkovich et al. Nature 330:440-450 (1987); and Zelent et al., Nature 339:714-717 (1989).

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Apart from their function as hormones in cellular growth and differentiation, retinoids are also involved in the visual process, as the stereo isomer 11-cis retinaldehyde is the chromophore of the visual pigments. See, e.g. Bridges, The Retinoids, Vol. 2, pp 125-176, Academic Press, Orlando, Florida, (1984).

30

Under normal physiological conditions most cells, both ocular and non-ocular, obtain all-trans retinol as their major source of retinoids. Despite the many different metabolic events taking place in different tissues, it is known that a common extracellular transport machinery for retinol has evolved.

5 Specifically, in plasma, retinol is transported by plasma retinol binding protein (RBP). See Goodman et al., The Retinoids, Academic Press, Orlando Florida, Volume 2, pp. 41-88 (1984). The active derivatives of retinol, retinoic acid in non-ocular tissues and mostly 11-cis retinaldehyde for ocular tissues, are then generated by cellular conversion using specific mechanisms. To date, none of

10 these mechanisms have been fully defined at the molecular level and several of the enzymes involved have only been identified by enzymatic activities. See Lion et al., *Biochem. Biophys. Acta.* 384:283-292 (1975); Zimmermann et al., *Exp. Eye Res.* 21:325-332 (1975); Zimmerman, *Exp. Eye Res.* 23:159-164 (1976) and Posch et al., *Biochemistry* 30:6224-6230 (1991).

15 Polarized retinal pigment epithelial cells (RPE) are unique with regard to retinoid uptake since all-trans retinol enters these cells via two different mechanisms. Retinol accumulated from RBP is taken up through the basolateral plasma membrane, while all-trans retinol, presumably taken up from the interstitial retinol-binding protein (IRBP) following bleaching of the

20 visual pigments, may enter through the apical plasma membrane. See Bok et al., *Exp. Eye Res.* 22:395-402 (1976); Alder et al., *Biochem. Biophys. Res. Commun.* 108:1601-1608 (1982); Lai et al., *Nature* 298:848-849 (1982); and Inu et al., *Vision Res.* 22:1457-1468 (1982).

The transfer of retinol from RBP to cells is a subject under

25 investigation. In a number of cell types, including RPE, specific membrane receptors for RBP have been identified, which is consistent with a receptor-mediated uptake mechanism for retinol. For example, isolated retinol binding protein receptors, nucleic acid molecule coding for these receptors and antibodies binding to the receptor are known.. These teachings relate to the

30 first of the two mechanisms. See Bavik et al., *J. Biol. Chem.* 266:14978-14985 (1991); Bavik, et al. *J. Biol. Chem.* 267:23035-23042 1992; Bavik et

al., J. Biol. Chem. 267:20540-20546 (1993); and U.S. Patent Nos. 5,573,939 and 5,679,772, all of which are incorporated by reference. See also Heller, J. Biol. Chem. 250:3613-3619 (1975); and Bok et al., Exp. Eye Res. 22:395-402 (1976).

5 Retinol uptake on the apical side of the RPE for the regeneration of 11-cis retinaldehyde ("11-cis retinal" hereafter) is less well characterized. However, regardless of the origin of all-trans retinol, the synthesis and apical secretion of 11-cis retinal seems to be the major pathway for accumulated retinol in the RPE. At present, it is not known whether similar mechanisms are
10 used with regard to cellular retinol uptake through the basolateral and the apical plasma membranes. However, available data show that functional receptors for RBP are exclusively expressed on the basolateral plasma membrane of RPE-cells. Bok et al., Exp. Eye Res. 22:395-402 (1976).

 It is also known that retinal pigment epithelial cells (RPE) express
15 a 63 kDa protein (p63). It has also been shown by chemical cross-linking that this protein may be part of an oligomeric protein complex which functions as a membrane receptor for plasma retinol-binding protein (RBP) in RPE-cells, or a component of the retinoid uptake machinery in RPE cells. See Bavik et al., J. Biol. Chem. 266:14978-14875 (1991); Bavik et al., J. Biol. Chem.
20 267:23035-23042 (1992), and U.S. Patent Nos. 5,573,939 and 5,679,772. The p63 protein has been isolated and the corresponding cDNA cloned. See Bavik et al., J. Biol. Chem. 267:20540-20546 (1993) and the '939 and '772 patents referred to supra. All of these references are incorporated by reference.

25 11-cis retinal, referred to supra is important in vision, because it is the light sensing chromophore found in cone opsins and rod opsins (i.e., "rhodopsin"), in both cone and rod photoreceptor cells. Deficiencies in vitamin A result in reduction in concentrations of rhodopsin in the retina, which is followed by night blindness. In turn, if night blindness is left untreated it is
30 followed by degeneration of rod photoreceptors, and then cone photoreceptors. In fact, vitamin A supplementation has been reported to slow

the course of retinal degeneration in diseases such as retinitis pigmentosa (Berson et al, Arch. Ophthalmol 111:761-772(1993), and to reverse the night blindness found in Sorsby fundus dystrophy, at least temporarily. See Jaconson, et al, Nature Genet. 11:27-32(1995).

5 Deficiencies in vitamin A can be attributed to one or more causes, including poor diet, or a deficiency in one or more of the proteins involved in transport of vitamin A through the bloodstream. See, e.g., Wetterau, et al., Science 258:999-1001 (1992), and Narcisi, et al., Am. J. Hum. Genet 57:1298-1310 (1995), discussing an inherited deficiency in microsomal
10 triglyceride transfer protein, and Seeliger, et al., Invest. Ophthal Vis. Sci. 40:3-11 (1999), discussing an inherited deficiency in serum retinol binding protein.

Physiological abnormalities and visual symptoms also arise from defects in the storage or metabolism of vitamin A within the retina. With respect to the storage of the vitamin, a number of proteins are thought to bind
15 11-cis and all-trans vitamin A alcohols and aldehydes in the retina and the retinal pigment epithelium. These proteins include "CRALBP", or cellular retinaldehyde binding protein, "IRBP", or "inter-photoreceptor retinoid binding protein", and "CRBP", or cellular retinol-binding protein. CRALBP and IRBP are known to be essential to photoreceptor physiology, since *null* mutations in
20 the genes encoding these proteins cause photoreceptor degeneration in mammals. See Mau, et al., Nature Genet 17:198-200 (1997); Morimura, et al., Invest. Ophthalmol. Vis. Sci. 40:1000-1004 (1999); Burstedt, et al., Invest. Ophthal. Vis. Sci. 40:995-1000 (1999); Liou, et al., J. Neurosci 18:4511-4520 (1998).

25 In contrast to the understanding of the pathways and mechanisms discussed supra, abnormalities in the metabolic pathways which convert all-trans retinol from the bloodstream into 11-cis retinal, and that reconvert the all-trans retinal produced after cone and rod photopigments absorb photons of light back to 11-cis retinol are not well understood. Various enzymes are
30 involved in this pathway, and are found in photoreceptor cells, as well as neighboring retinal pigment epithelium, and Müller cells of the retina. Several

of these enzymes have been purified only recently. For example, see Simon et al., J. Biol. Chem 270:1107-1112 (1995), and U.S. Patent No. 5,731,195, both of which are incorporated by reference for teachings relating to purified 11-cis retinal dehydrogenase, and molecules encoding this enzyme, Haeseleer, et al., J. Biol. Chem 273:21790-21799 (1998), for teachings related to all-trans retinal oxido reductase, and Ruiz, et al., J. Biol. Chem 274:3834-3841 (1999), for teachings relating to lecithin: retinal acyltransferase.

It has now been found that ocular disorders are associated with mutations in the nucleic acid molecules encoding 11-cis retinal dehydrogenase (RDH5), and the resulting mutated proteins. These are features of the invention which are set out in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows gel analysis of DNA taken from patients, showing mutation at codon 238 of the RDH5 gene.

Figure 2 shows gel analysis of DNA taken from patients, showing mutations at codons 238 and 73

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

EXAMPLE 1

Given the role of 11-cis retinol dehydrogenase (SEQ.ID NO: 1) in vision, it was assumed that patients suffering from hereditary retinal degeneration or malfunction might provide a source of mutated RDH5 genes. Patients with diseases featuring subretinal white or pale yellow spots were chosen for study, because these spots can arise from a lack of vitamin A, and are also found in patients with hereditary retinal degeneration caused by a lack of serum retinol binding protein (Seeliger et al., Invest. Ophthalmol Vis. Sci. 40:3-11 (1999)), or CRALBP (Morimura, et al., Invest. Ophthalmol Vis. Sci. 40:1000-10004 (1999)); Burstedt, et al., Invest. Ophthalmol Vis. Sci. 40:995-1000 (1999)). Twenty nine unrelated patients were evaluated who exhibited retinal

degeneration with subretinal spots (retinitis punctata albescens, or albipunctate dystrophy), two patients were evaluated who suffered from night blindness and subretinal spots (fundus albipunctatus), as were 94 normal controls, 71 patients with recessive retinitis pigmentosa, and 73 with dominant retinitis pigmentosa.

- 5 The latter two groups were used because there were no assurances that the assumption underlying the study, i.e., that mutations would be found in the RDH5 gene of patients who exhibited subretinal spots, would be correct.

The RDH5 gene contains 4 translated exons. Exon 1 is not translated. Single strand confirmation analysis was used to screen the 4 translated exons,
10 as well as flanking intron sequences.

Genomic DNA was analyzed via polymerase chain reaction. Primers were based upon the sequence of RDH5 published by Simon et al., Genomics 36:424-430 (1996), and Gu, et al., (GenBank Acc. No. AF037062), both of which are incorporated by reference and SEQ ID NO: 2. The primer pairs
15 employed were:

5'- GGCCACAGTA AACTGGACAA-3'
(nucleotides 2301-2320 of SEQ ID NO:2)(sense)

20

5'- AGCCGGTGAT GAAGACAAAG-3'
(Nucleotides 2458-2477 of SEQ ID NO:2)(antisense)

which amplify exon 2a of the RDH5 gene;

25

5'- TTA CTCTGGG CAGTGCTGTG-3'
(nucleotides 2399-2418 of SEQ ID NO:2)(sense)

5'- AGGACTCGGA AGCCTCTCTG-3'
30 (nucleotides 2519-2538 of SEQ ID NO:2)(antisense)

which amplify exon 2b;

5'- TTCTGGCACT GCAGCTGGAC-3'
(nucleotides 2499-2518 of SEQ ID NO:2)(sense)

5

5'- TTCCTGGTGG TCTACCATAC-3'
(nucleotides 2686-2705 of SEQ ID NO:2)(antisense)

which amplify exon 2c;

10

5'- CCCCAGCATC CTTTTCATCT-3'
(nucleotides 2848-2867 of SEQ ID NO:2)(sense)

5'- GACGCTGGTG ATGTTGATCA-3'
15 (nucleotides 3038-3057 of SEQ ID NO:2))(antisense)

which amplify exon 3a;

5'- TGAACACAAT GGGTCCCATC-3'
20 (nucleotides 2969-2988 of SEQ ID NO:2)(sense)

5'- TGTTAGTCCT GGAACCCAGG-3'
(nucleotides 3151-3170 of SEQ ID NO:2)(antisense)

25 which amplify exon 3b;

5'- AAGAACCCAG CAACTTCGCT-3'
(nucleotides 4030-4049 of SEQ ID NO:2)(sense)

30 5'- TTCCCTTCAT GTGCCCTGT-3'
(nucleotides 5247-5266 of SEQ ID NO:2)(antisense)

which amplify exon 4;

5'- CTGATTGCAA CCACCTATGG-3'
(nucleotides 5473-5492 of SEQ ID NO:2)(sense)

5

5'- AGAGCAGCTT GGCATCCCAA-3'
(nucleotides 5619-5639 of SEQ ID NO:2)(antisense)
which amplify exon 5a; and

10 5'- TAACCAAGGT GAGCCGATGC-3'
(nucleotides 5549-5568 of SEQ ID NO:2)(sense)

5'- CAATCTCTTG CTGGAAGGCT-3'
(nucleotides 5731-5748 of SEQ ID NO:2)(antisense)

15 which amplify exon 5b.

Amplification of exon fragments was carried out by isolating DNA from leukocytes of subjects, and then adding 20-100 ng of the DNA to 20 μ l of a solution containing 20 pM of each pair of the primers described supra, 20 mM
20 Tris-HCl (pH 8.4), from 0.5 to 1.5 mM $MgCl_2$ (explained infra); 50 mM KCl, 0.02 mM of each of dATP, dTTP, and dGTP, and 0.002 mM of dCTP, 0.6 μ Ci [α - ^{32}P] dCTP (3000 Ci/mmol), 0.1 mg/ml bovine serum albumin, 10% DMSO (except for assays on exons 4 and 5a), and 0.25 units of Taq polymerase.

The amount of $MgCl_2$ varied, depending on the primer pair being used,
25 to obtain optimal amplification. In the assays where exons 2a, 2c and 3b were amplified, 0.5 mM was used. When amplifying exons 2b, 3a, and 5a, 1.0 mM was used, and 1.5 mM was used when amplifying exons 4 and 5b. Samples were heated to 94°C for 5 minutes to denature the double stranded DNA, and then from 22-30 cycles of amplification were carried out, with a cycle being
30 defined as 30 seconds of denaturing at 94°C, 30 seconds at 50-60°C for primer annealing, and 30 seconds of extension at 71°C. More specifically, the

annealing temperature for exons 2a,3a, and 5a was 58°C, it was 50 °C for exons 2c and 3b, and 60° C for 2b and 5b The final extension involved heating at 71°C for 5 minutes. In all cases, the pH of the reaction was 8.4.

Amplified DNA was heat denatured, using standard methods, and
5 aliquots of the resulting single stranded fragments were separated through two sets of 6% polyacrylamide gels. One set contained 10% glycerol, and the other did not. Amplification products of the assay for exon 2c were also evaluated by electrophoresis through MDE (mutation detection enhancement) gels. The electrophoresis was carried out for 5-18 hours, at room temperature, and at 8-
10 12 W, before drying and autoradiography. Variant bands (defined as bands migrating at a faster or slower than normal speed through any one of the gels) were evaluated by sequencing the corresponding PCR amplified segments, using standard methods.

The two patients with fundus albipunctatus were found to have missense
15 changes. Specifically, one patient was homozygous for a change in exon 4 at codon 238 (GGG to TGG),(nucleotides 5207-5209 of SEQ ID NO:2) leading to a change in the amino acid encoded by the gene (a change from Gly to Trp), while a second patient was heterozygous for the same change, and for a second change in exon 2 at codon 73 (TCC to TTC)(nucleotides 2589-2591 of
20 SEQ ID NO:2), leading to a change in the amino acid encoded (Ser to Phe). One patient with dominant retinitis pigmentosa exhibited a missense change in exon 2 at codon 33 (ATC to GTC)(nucleotides 2468-2470 of SEQ ID NO:2), leading to an amino acid change (Ile to Val). Silent polymorphisms were also found, at codon 141 (ATC to ATT)(nucleotides 2986-2988 of SEQ ID NO:2)
25 and codon 200 (GTC to GTG)(nucleotides 5093-5095 of SEQ ID NO:2).

The missense changes at 238 and 73 were studied further via familial analysis. For both subjects, the missense change segregated as would be expected if they caused the disease. For example, a sibling of the patient homozygous for the 238 missense change was also homozygous for the
30 change, and was afflicted with the disease, while the patient's mother and two unaffected siblings were heterozygous. With respect to the patient who was

heterozygous for the missense changes at 238 and 73, there were no affected relatives; however, an unaffected brother was heterozygous for the 238 missense change, and his mother was heterozygous for the 73 missense change. These data are set forth at Figures 1 and 2. It can be seen in Figure 1, for example, afflicted individuals are homozygous for a change at position 238, where "G" has been replaced by "T" in both alleles. In contrast, non-afflicted familial individuals are heterozygous for "G" and "T" at this position. The data in Figure 2 show that individuals can be "compound heterozygotes" in that there are single mutations at both positions 238 and 73. What this indicates is that compound heterozygosity can lead to the condition.

The results show that a homozygous mutation at position 238 is indicative of the condition, i.e., fundus albipunctatus. Heterozygous mutations, i.e., situations where only one allele carried the mutation, did not result in the mutation. On the other hand, the data also evidence compound heterozygosity, in that more than one, heterozygous mutation, results in an abnormal condition.

EXAMPLE 2

These experiments describe studies to determine the effect of the missense mutations described supra, i.e., Gly238Trp and Ser73Phe on the activity of 11-cis retinol dehydrogenase.

The first set of experiments was designed to generate mutant forms of the enzyme in vitro.

Human cDNA encoding 11-cis retinol dehydrogenase (RDH5)a is known, as per Simon, et al., supra, and allowed U.S. Patent Application Serial No. 08/258,418, filed June 10, 1994, and PCT WO95/34580 published May 29, 1997, as SEQ ID NO: 14, all of which are incorporated by reference. See SEQ ID NO: 5 as well. The human cDNA molecule described in these references was subcloned into eukaryotic expression vector pSG5, described by Green, et al., Nucl. Acids Res. 16:369 (1988), incorporated by reference. Expression vectors which encoded mutant forms of the enzyme were then generated using single strand mutagenesis, in accordance with Kunkel, et al., Methods

Enzymol. 154:367-382 (1987), and Viera, et al., Methods Enzymol. 154:3-11 (1987), both of which are incorporated by reference. The following primers were used to generate the Ser73Phe and Gly238Trp mutants, respectively:

5 5'- CTGCAGCGGG TGGCCTTTCTC CCGCCTCCAC
ACC-3'
(SEQ ID NO: 3)

10 5'- ACACAGGCCC ACTATTGGGG GGCCTTCCTC
ACC-3'
(SEQ ID NO: 4)

In SEQ ID NOS: 3 and 4, codons for the missense mutations are underlined.

15 Following verification that plasmids carried the introduced mutations, these were used to separately transfect COS-1 cells. As controls, expression vectors expressing wild type 11-cis-retinol dehydrogenase (RDH5), or an empty vector were used. All transfections were carried out using the well known DEAE-dextran methodology, as described by Simon, et al., J. Biol. Chem.
20 270:1107-1112 (1995), incorporated by reference. An expression vector which expressed β -galactosidase (pSV β gal), which is commercially available, was cotransfected in each experiment. The transfectants were cultured for 48 hours, under standard conditions, after which they were harvested. Microsomes were prepared from these cells, via gentle homogenization and
25 centrifugation at 7000 x g for 10 minutes, in order to remove debris, and unbroken cells.

Microsomes were collected from supernatant via centrifugation at 100,000 x g for 60 minutes. The microsomes were suspended in PBS, protein concentrations were determined using standard methods, and then aliquots
30 were stored at - 80°C, until used. Equal efficiencies of the transfections were

verified by measuring β -galactosidase activity using o-nitrophenyl- β -D-galactopyranoside at 405 nm.

Expression levels of wild type and mutant enzymes were determined via immunoblotting, using pre-existing polyclonal rabbit antiserum, in accordance with the ECL methodology as described by Simon, et al., J. Cell Sci. 112:549-558 (1999).

It was ascertained that both mutants were expressed at levels 8-12 times lower than wild type enzyme.

To monitor the ability of the wild-type and mutant enzymes to catalyze the oxidation of 11-cis retinol to 11-cis retinal, 11-cis retinol, which had been synthesized by reduction of 11-cis retinal with NaBH₄ and stored under argon at -80°C, was added to reaction mixtures containing microsomes from the transfected cells. Microsomes containing the wild-type enzyme exhibited the expected ability to catalyze the oxidation of 11-cis retinol to 11-cis retinal in the presence of an excess of the cofactor NAD.

The enzyme reactions, in a total volume of 100 μ l were carried out in 50mM Tris-HCl buffer pH7.5 containing 5mM NAD or NADH, 50 M 11-cis retinol, or 11-cis retinal, and microsomes at 37 °C. All manipulations of retinoids were performed in dim light. The reactions were stopped by putting the tubes on ice, and the retinoids were immediately extracted with 200 μ l n-hexane. Subsequently, 75 μ l aliquots were analyzed by reverse-phase HPLC using a C18 column. The mobile phase was acetonitrile/water (85/15, v/v), and the column was eluted under isocratic conditions at a flow rate of 1ml/min. 11-cis retinol and 11-cis retinal eluted at 12.0-12.2 and 14.0-14.3 minutes, respectively. Retinoids were quantified at 320 and 370nm. To measure the K_m and V_{max} values of wild type 11-cis retinol dehydrogenase, reactions were carried out as above, using a concentration of 11-cis retinol between 0.5 μ M and 50 M. In these assays, 2 μ g of microsomal protein containing the wild-type enzyme were used in 8-minute incubations. Formed 11-cis retinal was quantified in the HPLC analyses, and the K_m and V_{max} values were calculated

from Lineweaver-Burk plots. The values were compensated for the extraction efficiency (60-70%, as determined experimentally).

The K_m and V_{max} values for 11-cis retinol in this reaction were determined to be 6.7 M (average of duplicates 5.5 and 7.8), and 8.4 nmol/mg protein/minute (average of duplicates 7.1 and 9.6), respectively. These results are consistent with a report by Wang, et al., *Biochem. J.* 338:23(1999). As expected, the wild-type enzyme was also able to catalyze the reverse reaction, i.e., the reduction of 11-cis retinal to 11-cis retinol, in the presence of an excess of NADH.

The relative activities of the wild type and mutant enzymes were then determined. To do this, microsomes containing a mutant enzyme (20 μ g), or wild type enzyme (2 μ g) together with 18 μ g of microsomal protein from mock-transfected cells, to standardize the experimental conditions, or no enzyme were combined with 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM of NAD, and 50 μ M 11-cis-retinol (forward, oxidation reaction) or 5 mM NADH and 50 μ M 11-cis-retinal (reverse, reduction reaction) in a total volume of 100 μ l. 50 μ M 11-cis retinol is approximately a seven-fold higher substrate concentration than the estimated K_m value for the wild-type enzyme. The mixtures were incubated for 10 minutes at 37°C, after which the reactions were stopped by putting the mixtures on ice. Retinoids were extracted immediately with 200 μ l of n-hexane. Subsequently, 75 μ l aliquots were analyzed via reversed phase HPLC using a C_{18} column. The mobile phase was acetonitrile/water (85:15 v/v), and the column was eluted under isocratic conditions at a flow rate of 1 ml/min. The 11-cis-retinol eluted at 12.0-12.2 minutes, and 11-cis-retinal at 14.0-14.3 minutes. Retinoids were quantified at 320 (retinol) and 370 (retinal) nm. Relative activities of wild-type and mutant enzymes were calculated as the ratio of formed 11-cis retinal by transfected versus mock-transfected microsomes. The results were normalized for the total extracted retinoid content from each reaction.

The wild type enzyme exhibited the expected ability to catalyze the oxidation of 11-cis-retinol to 11-cis-retinal in the presence of an excess of NAD.

Similarly, it catalyzed the reverse reaction, i.e., reduction of 11-cis-retinal to 11-cis-retinol, in the presence of excess NADH.

Both mutants exhibited dramatic reductions in their ability to catalyze the relevant reactions, even though they were used at 10 fold higher concentrations in view of their lower expression levels. The Gly238Trp mutant showed no activity above background level, while the Ser73Phe mutant showed residual activity. Similar results were obtained when catalyzing the reverse reaction

The Gly238Trp mutant was tested further, to determine if it had any activity, by incubating for 20 and 40 minute periods before extracting retinoids. Wild type and Ser73Phe mutant were used as controls. The Gly238Trp mutant showed 2.2 ± 0.14 fold activity above background activity (using mock transformants), as compared to 42.9 ± 4.5 fold (wild type), and 9.1 ± 1 fold (Ser273Phe), after 40 minutes. This indicates that the Ser273Phe mutant has approximately 5 fold less activity, and the Gly238Trp mutant approximately 19 fold less activity than wild type enzyme.

The foregoing examples describe the invention, which relates to mutations in the nucleic acid molecule which encodes 11-cis retinol dehydrogenase, as well as the resulting mutated protein. Specifically, mutations at the codon which encodes amino acid 238 and/or the codon which encodes amino acid 73 are a feature of the invention. In particular, with respect to codon 238-Trp, rather than Gly, is encoded, and with respect to amino acid 73Phe, rather than Ser, is encoded. Also a feature of the invention are molecules where the mutation is at position 33, especially those where Val, rather than Ile is encoded. In addition to the specific mutations described supra, it will be understood by the skilled artisan that, in view of codon degeneracy, more than one type of mutation may result in the specific changes.

As was explained, supra, the mutated forms of the encoding molecules and the proteins are involved in ocular disorders. Hence, a further feature of this invention is the determination of the possible presence of an ocular

disorder, via determining presence of a mutated form of either the nucleic acid molecule, the protein, or both. Such methods include, e.g., hybridization assays such as the polymerase chain reaction, antibody assays involving antibodies specific for an epitope defined by the mutation, and so forth. One
5 specific type of disorder which may be determined via these methodologies is fundus albipunctatus.

Fundus albipunctatus is a form of night blindness wherein patients who suffer from rod photoreceptor malfunction recover after prolonged exposure to darkness. This condition is characterized further by delays in recovery of both
10 rod and cone function after exposure to light; however, fundus albipunctatus is characterized as a form of night blindness, because cone recovery from exposure to light is generally not sufficiently impaired to be subjectively important.

The experiments set forth supra show that the abnormally slow rates of
15 regeneration of cone and rod photopigment found in fundus albipunctatus result from slower than normal rates of production of 11-cis retinal, due to reduced 11-cis retinol dehydrogenase activity. Further, decreased, steady state levels of mutant enzymes were seen, suggesting folding or stability problems. Indeed, these two phenomena point to a net capacity of the enzyme
20 to generate 11-cis retinal at one order of magnitude lower than normal. These observations suggest a further application of the invention, which is the treatment of conditions characterized by mutations in the gene encoding 11-cis retinal dehydrogenase, or mutated forms of the protein, by administration of an amount of normal, or "wild type" 11-cis retinol dehydrogenase sufficient to
25 alleviate the disorder. Similarly, since the enzyme is involved in the production of 11-cis retinal, the therapy can also take the form of administration of 11-cis-retinal, in any of the standard forms of ocular administration. Similarly, principles of gene therapy, such as homologous recombination, can be employed to correct the mutation, upon its detection

30 As the mutated forms of the enzyme are useful in the ways discussed supra, it is desirable to have a ready source of the materials. Hence, another

feature of this invention is the recombinant production of the enzyme, via the use of transformed or transected cells, where the resulting recombinant cells produce the mutant enzyme, as well as multiple copies of the desired nucleic acid molecule.

- 5 Mutations in the nucleic acid molecule, in addition to those described above, i.e., at codons for amino acids 238, or 73, or 33, which result in inactive or less active forms of the enzyme, are also a part of this invention. These mutations can include other missense changes, as well as deletions, insertions, frame shift mutations, mutations affecting intron splice donor or acceptor sites,
10 mutations in the promotor region, and so forth. Identification of such mutations via, e.g., PCR, or other forms of assays, are indicative of possible presence of an optical disorder.

Other features of the invention will be clear to the skilled artisan, and are not set forth herein.

- 15 The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

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5 WE CLAIM:

1. An isolated protein comprising the amino acid sequence of wild type retinol dehydrogenase as set forth in SEQ ID NO: 1, with the proviso that (i) amino acid 238 is not Gly or (ii) amino acid 73 is not Ser, or (iii)
10 amino acid 33 is not Ile.
2. The isolated protein of claim 1, wherein amino acid 238 is Trp rather than Gly.
- 15 3. The isolated protein of claim 1, wherein amino acid 73 is Phe rather than Ser.
4. The isolated protein of claim 1, wherein amino acid 33 is Val rather than Ile.
20
5. An isolated nucleic acid molecule which encodes the protein of claim 1.
6. An isolated nucleic acid molecule which encodes the protein of claim 2.
- 25 7. An isolated nucleic acid molecule which encodes the protein of claim 3.
8. An isolated nucleic acid molecule which encodes the protein of claim 4.
9. Expression vector comprising the isolated nucleic acid molecule of claim
30 5, operably linked to a promoter.

10. Recombinant cell comprising the isolated nucleic acid molecule of claim 1.
11. Recombinant cell comprising the expression vector of claim 9.
- 5 12. A method for determining possible presence of a disorder of the eye, comprising assaying a sample taken from a subject believed to have an eye disorder for a mutation in the nucleic acid molecule which encodes retinol dehydrogenase, presence of said mutation being indicative of possible presence of said disorder.
- 10 13. The method of claim 12, said method comprising polymerase chain reaction.
- 15 14. The method of claim 12, wherein said mutation is a mutation at the codon which encodes amino acid 73 of retinol dehydrogenase.
- 15 15. The method of claim 12, wherein said mutation is a mutation at the codon which encodes amino acid 238 of retinol dehydrogenase.
- 20 16. The method of claim 15, wherein said disorder is fundus albipunctatus.
17. The method of claim 16, wherein said mutation occurs in both alleles of said subject's gene which encodes retinol dehydrogenase.
- 25 18. The method of claim 17, wherein both of said alleles carry the same mutation.
- 30 19. The method of claim 17, wherein each of said alleles carries a different mutation

20. The method of claim 18, wherein said mutation results in a change from glycine to tryptophan at the codon for amino acid 238.

5 21. The method of claim 19, wherein one of said alleles carries a mutation resulting in a change from glycine to tryptophan at the codon for amino acid 238, and the other allele carries a mutation resulting in a change at the codon for amino acid 73, resulting in a change from serine to phenylalanine.

10

FIG. 1

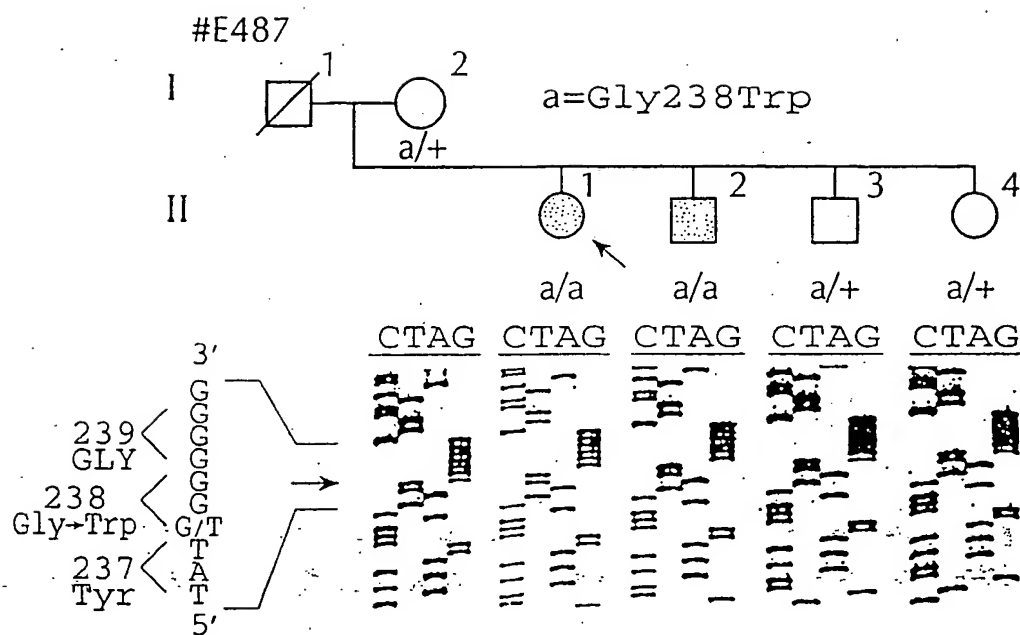
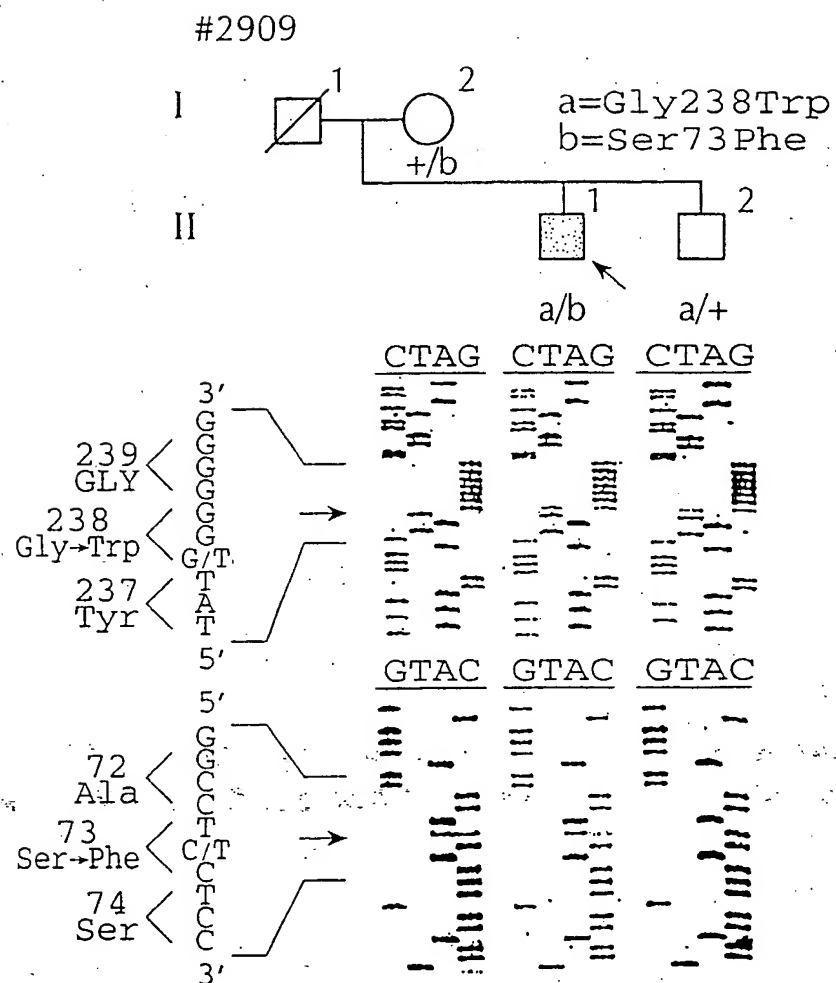


FIG. 2



SEQ ID NO: 1

Met	Trp	Leu	Pro	Leu	Leu	Leu	Gly	Ala	Leu	Leu	Trp	Ala	Val	Leu	Trp
			5						10					15	
Leu	Leu	Arg	Asp	Arg	Gln	Ser	Leu	Pro	Ala	Ser	Asn	Ala	Phe	Val	Phe
			20					25					30		
Ile	Thr	Gly	Cys	Asp	Ser	Gly	Phe	Gly	Arg	Leu	Leu	Ala	Leu	Gln	Leu
		35					40					45			
Asp	Gln	Arg	Gly	Phe	Arg	Val	Leu	Ala	Ser	Cys	Leu	Thr	Pro	Ser	Gly
	50					55					60				
Ala	Glu	Asp	Leu	Gln	Arg	Val	Ala	Ser	Ser	Arg	Leu	His	Thr	Thr	Leu
65					70					75					80
Leu	Asp	Ile	Thr	Asp	Pro	Gln	Ser	Val	Gln	Gln	Ala	Ala	Lys	Trp	Val
				85					90					95	
Glu	Met	His	Val	Lys	Glu	Ala	Gly	Leu	Phe	Gly	Leu	Val	Asn	Asn	Ala
			100					105					110		
Gly	Val	Ala	Gly	Ile	Ile	Gly	Pro	Thr	Pro	Trp	Leu	Thr	Arg	Asp	Asp
		115					120					125			
Phe	Gln	Arg	Val	Leu	Asn	Val	Asn	Thr	Met	Gly	Pro	Ile	Gly	Val	Thr
	130					135					140				
Leu	Ala	Leu	Leu	Pro	Leu	Leu	Gln	Gln	Ala	Arg	Gly	Arg	Val	Ile	Asn
145					150					155					160
Ile	Thr	Ser	Val	Leu	Gly	Arg	Leu	Ala	Ala	Asn	Gly	Gly	Gly	Tyr	Cys
				165					170					175	
Val	Ser	Lys	Phe	Gly	Leu	Glu	Ala	Phe	Ser	Asp	Ser	Leu	Arg	Arg	Asp
			180					185					190		
Val	Ala	His	Phe	Gly	Ile	Arg	Val	Ser	Ile	Val	Glu	Pro	Gly	Phe	Phe
		195					200					205			
Arg	Thr	Pro	Val	Thr	Asn	Leu	Glu	Ser	Leu	Glu	Lys	Thr	Leu	Gln	Ala
	210					215					220				
Cys	Trp	Ala	Arg	Leu	Pro	Pro	Ala	Thr	Gln	Ala	His	Tyr	Gly	Gly	Ala
225					230					235					240
Phe	Leu	Thr	Lys	Tyr	Leu	Lys	Met	Gln	Gln	Arg	Ile	Met	Asn	Leu	Ile
				245					250					255	
Cys	Asp	Pro	Asp	Leu	Thr	Lys	Val	Ser	Arg	Cys	Leu	Glu	His	Ala	Leu
			260					265					270		

Thr Ala Arg His Pro Arg Thr Arg Tyr Ser Pro Gly Trp Asp Ala Lys
 275 280 285

Leu Leu Trp Leu Pro Ala Ser Tyr Leu Pro Ala Ser Leu Val Asp Ala
 290 295 300

Val Leu Thr Trp Val Leu Pro Lys Pro Ala Gln Ala Val Tyr
 305 310 315

SEQ ID NO: 2

1 ccaggttttc cctcccttcc ccactcagc tgcaggaact cctttttggg gtttgatct
 61 ggtatttttc tttcagctc cgagcttggc tctcctggg aatcctggga gtgaaaggaa
 121 ggagctgggt ttatttgcac gtactggtag tcatttgcac cacatccaaa aatggccaaa
 181 attataaccc ctgattcttg gctgaactgg gactgctgca atggaatatt attcccggaa
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 361 caatttgcca agcaaacagg ccattggatc gaaatgggtg aaaacttcaa ccaggcactc
 421 aaggtggggc atactcccta cctcaccacc ccaatcctgg gccccattg gctgcctcca
 481 gtcaggttac ctcaggttta ggtaaggag gaagtagggt ggtcccagaa accccatcta
 541 tagcccccag gtcagaaaag gttgagaaag aaagaaaagc agttggtggg tccaagttaa
 601 agccttttcc aggagatgaa taaaacttat tccccaatgg aagcctatgt ctaccattc
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 721 cttggagcat tgagctggaa atgctcacca ttgccaatgc aatggaatat gtttacaaag
 781 ggcagctgca gtttgccctc tctagcccc tggtccctcc cccaacccta tccctcctac
 841 ctcacccgca gggggaagga gggaggctga caagccttga ataaaaaaca agcctccgtt
 901 tttttgtggt gtgtttcaga gaggtaatag ctccagtgtc gggggtggga gtggaagggt
 961 caaaggtggt ttccctgagg gacaggtacc ttttggggag aggttgaaa tagcttcctt
 1021 ttactatccc aaattttttt tcttccatgg cccttggtga ggtgtttgtt aggcaagcag
 1081 aggggtgggag ttcccatccc tcttgagaga aggtcctagt agcctgccc caagcttctt
 1141 aattcaggaa ttgtttccta cagaagagaa acaaggcaag tacacctggt cccagctct
 1201 ggctttctgc ctctccacgt gctcatggcc tctcccagg ctaactctaa gcagtgtcat
 1261 gagtctgagc caggtgggag attaattcct gggggcactt cagggctgag aagggggagg
 1321 aatgacaggt ccagtaaccg ttaccaacag agcagtgcag ctgccatcct tgacagctcc
 1381 ctctccttg gagaccatga catagatggt caggaaccca ggctgagaaa gacagccaag
 1441 gggtaggggg agcctaggca aatctggcct ctgccaagtc ctggcttcag ccaggcaagc
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6181 aggcaatatt cacataaatt ctctgtctaa ggacactgac cacacagggt tgcaaggcaa
6241 cttatcatac ttcgaaagga gctggatccc ttgaggattg gccaggaagg gaggtgctgg
6301 gcccttagcg gtgcacagaa ggccagggaag

SEQ ID NO: 5

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60

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120

GTGGTTGCTC AGGGACCGGC AGAGCCTGCC CGCCAGCAAT GCCTTTGTCT TCATCACCGG
180

CTGTGACTCA GGCTTTGGGC GCCTTCTGGC ACTGCAGCTG GACCAGAGAG GCTTCCGAGT
240

CCTGGCCAGC TGCCTGACCC CCTCCGGGGC CGAGGACCTG CAGCGGGTGG CCTCCTCCCG
300

CCTCCACACC ACCCTGTTGG ATATCACTGA TCCCCAGAGC GTCCAGCAGG CAGCCAAGTG
360

GGTGGAGATG CACGTTAAGG AAGCAGGGCT TTTTGGTCTG GTGAATAATG CTGGTGTGGC
420

TGGTATCATC GGACCCACAC CATGGCTGAC CCGGGACGAT TTCCAGCGGG TGCTGAATGT
480

GAACACAATG GGTCCCATCG GGGTCACCCT TGCCCTGCTG CCTCTGCTGC AGCAAGCCCG
540

GGGCCGGGTG ATCAACATCA CCAGCGTCCT GGGTCGCCTG GCAGCCAATG GTGGGGGCTA
600

CTGTGTCTCC AAATTTGGCC TGGAGGCCTT CTCTGACAGC CTGAGGCGGG ATGTAGCTCA
660

TTTTGGGATA CGAGTCTCCA TCGTGGAGCC TGGCTTCTTC CGAACCCTG TGACCAACCT
720

GGAGAGTCTG GAGAAAACCC TGCAGGCCTG CTGGGCACGG CTGCCTCCTG CCACACAGGC
780

CCACTATGGG GGGGCCTTCC TCACCAAGTA CCTGAAAATG CAACAGCGCA TCATGAACCT
840

GATCTGTGAC CCGGACCTAA CCAAGGTGAG CCGATGCCTG GAGCATGCCC TGA CTGCTCG
920

ACACCCCGA ACCCGCTACA GCCCAGGTTG GGATGCCAAG CTGCTCTGGC TGCCTGCCTC
980

CTACCTGCCA GCCAGCCTGG TGGATGCTGT GCTCACCTGG GTCCTTCCCA AGCCTGCCCA
1020

AGCAGTCTAC TGAATCCAGC CTTCCAGCAA GAGATTGTTT TTCAAGGACA AGGACTTTGA
1080

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1128